EXHIBIT R

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Proangiogenic role of tumor-activated hepatic stellate cells in experimental melanoma metastasis.

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Myofibroblasts infiltrate malignant liver tumors, although their pathogenic implications are unclear. Immunohistochemical detection of alpha-smooth muscle actin, glial fibrillary acidic protein (GFAP), and CD31 and CD34 expression was used to analyze the contribution of myofibroblasts to angiogenesis in hepatic metastasis produced by intrasplenically-injected B16 melanoma (B16M). Because activated hepatic stellate cells (HSCs) are oxygen-sensing myofibroblasts producing vascular endothelial growth factor (VEGF), the effect of B16M and human A375 melanoma supernatants on VEGF production by immortalized rat HSC line T6 and primary cultured human HSCs also was studied under an hypoxic atmosphere mimicking a tumor microenvironment. Myofibroblast infiltration preceded endothelium recruitment in avascular micrometastasis and generated specific stroma for sinusoidal-type and portal-type angiogeneses. Thereafter, myofibroblasts and endothelial cells colocalized within both angiogenic patterns and their numerical densities correlated with metastasis development. Myofibroblasts often were GFAP-positive, suggesting an HSC origin. Melanoma supernatants stimulated VEGF messenger RNA and protein synthesis by HSCs. These effects were potentiated by hypoxia. VEGF up-regulation was accompanied by increased expression of cyclooxygenase type 2 (COX-2) and PGE2 synthesis. HSC production of VEGF decreased under COX-2 inhibition, whereas it was increased by exogenous PGE2. The high VEGF expression in HSCs induced by melanoma factors and hypoxia resulted in mitogenic, antiapoptotic, and motogenic stimulation of both murine hepatic sinusoidal endothelium and human umbilical vein endothelium. In conclusion, temporal and positional relationships evolve between myofibroblast and endothelium recruitment during metastasis development. Mechanistically, hypoxic induction of VEGF in tumor-activated HSCs may create a proangiogenic microenvironment, facilitating endothelial cell recruitment and survival during hepatic metastasis transition from an avascular to a vascular stage.

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Angiogenesis in cutaneous melanoma: pathogenesis and clinical implications.

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Neovacularization is an essential step in the multistage progression of malignant melanoma. The onset of new blood vessel formation is ushered in by the release of VEGF and numerous other angiogenic molecules by the tumor cells. Human melanoma is unique among neoplasms that both avascular (early horizontal growth phase characterized by very slow progression and 99%, 10-year survival) and vascular (late radial and vertical growth phase associated with rapid growth, metastasis and death in many cases), phases are discernible by the naked eye. Although cell biologists have made great strides in unraveling the mechanisms involved in the laying down of tumor vasculature and the factors that inhibit it, clinicians treating melanoma have been rather slow to realize and utilize the full potential of suppressing the tumor blood flow to the best advantage of the patient. We suggest a consorted endeavor by all the melanoma experts across the globe to establish an "angiogenesis database" wherein they pool the blood flow and vascularity information along with Breslow's thickness, Clark's level of invasion, lymphatic and vascular invasion, regression, and outcome of their patients. Copyright 2003 Wiley-Liss, Inc.